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Differences in B cell growth phenotype reflect novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells

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Recently established Epstein–Barr virus (EBV)-positive Burkitt's lymphoma (BL) cell lines, carrying chromosomal translocations indicative of their malignant origin, have been monitored for their degree of *in vitro* progression towards a more 'lymphoblastoid' cell surface phenotype and growth pattern, and for their expression of three EBV latent gene products which are constitutively present in all virus-transformed normal lymphoblastoid cell lines (LCLs). BL cell lines which stably retained the original tumour biopsy phenotype on serial passage were all positive for the nuclear antigen EBNA 1 but did not express detectable amounts of two other 'transforming' proteins, EBNA 2 and the latent membrane protein (LMP). This novel pattern of EBV gene expression was also observed on direct analysis of BL biopsy tissue. All three viral proteins became detectable, however, in BL cell lines which had progressed towards a more LCL-like phenotype *in vitro*. This work establishes a link between B cell phenotype and the accompanying pattern of EBV latent gene expression, and identifies a novel type of EBV:cell interaction which may be unique to BL cells.

Key words: Epstein–Barr virus/gene expression/B cell phenotype/Burkitt's lymphoma/latency

Introduction

The Epstein–Barr virus (EBV), found in all human communities as a widespread and largely asymptomatic infection, shows a strong association with two human B cell malignancies. One is Burkitt's lymphoma (BL), a tumour which in its high incidence ('endemic') areas of equatorial Africa and New Guinea is EBV genome-positive in more than 95% of cases (Lenoir and Bornkamm, 1986). The second is the B cell lymphoma to which immunologically-compromised individuals are particularly prone (Clearly *et al.*, 1986); this presents as an EBV-carrying oligoclonal proliferation strikingly similar to the disease induced experimentally by the virus in susceptible sub-human primates (Cleary *et al.*, 1985).

Most of our knowledge of EBV-induced B cell proliferation comes not from these malignancies, but from the capacity of the virus to transform normal resting B cells in culture into permanent lymphoblastoid cell lines (LCLs) in which every cell carries multiple episomal copies of the EBV genome (Henle *et al.*, 1967; Pope *et al.*, 1968). A restricted number of viral proteins, the so-called 'latent' viral gene products, are constitutively expressed in LCLs and appear to be collectively responsible for cell growth transformation. To date, these include the nuclear antigens EBNA 1 (Summers *et al.*, 1982) encoded by the BKRF1 reading frame (Baer *et al.*, 1984), EBNA 2 (Dambaugh *et al.*, 1984; Dillner *et al.*, 1985; Rowe,D. *et al.*, 1985) encoded by BYRF1, EBNA 3 (Hennessy *et al.*, 1986; Kallin *et al.*, 1986; Rowe,D. *et al.*, 1987) encoded by BLRF3 and BERF1 spliced together (Hennessy *et al.*, 1986; Joab *et al.*, submitted for publication), and leader protein (LP) encoded by highly spliced exons from the Bam WY region (Sample *et al.*, 1986; Dillner *et al.*, 1986; Speck *et al.*, 1986; Rowe,D. *et al.*, 1987). Whilst all of the EBNA species can be detected by naturally-occurring antibodies present in certain EBV-immune human sera, the presence of an additional latent gene product, the latent membrane protein (LMP), in EBV-transformed cells has only been demonstrated using antibodies raised experimentally to a relevant fusion protein (Hennessy *et al.*, 1984; Mann *et al.*, 1985) or to a synthetic peptide (Rowe,D. *et al.*, 1986).

Little is yet known of the functions of these viral proteins in the transformation process. EBNA 1, through binding to the *ori-P* sequence on the viral genome, plays a role in the maintenance and replication of episomal virus DNA (Yates *et al.*, 1984, 1985; Rawlins *et al.*, 1985) but may also have other regulatory functions. EBNA 2 has long been suspected as having a role in the initiation of transformation since rare EBNA 2-deletion mutants (Bornkamm *et al.*, 1982; Rabson *et al.*, 1982) cannot activate resting B cells into cycle (Miller *et al.*, 1974). Two families of natural EBV isolates have been identified with distinct EBNA 2 alleles which share less than 50% homology and which encode antigenically-distinct proteins, EBNA 2A and EBNA 2B (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985). Recent work has shown that the precise growth phenotype of an LCL is influenced by the EBNA 2 type of its resident virus (Rickinson *et al.*, 1987), a result which implies some continuing role for EBNA 2 in maintaining the transformed state of LCL cells. Of the other latent viral genes, the only available functional evidence relates to LMP which, when transfected and expressed in the Rat 1 cell line, converted the cells to anchorage independent growth in soft agar and to tumourigenicity in nude mice (Wang *et al.*, 1985); this implicates LMP as an important effector protein in human cell transformation.

The consistent pattern of EBV gene expression seen in LCLs is associated with an equally consistent cellular phenotype, characterised by high rate expression of a number of B cell 'activation' antigens on the cell membrane (Rowe,M. *et al.*, 1982; Thorley-Lawson *et al.*, 1985) and by a typical pattern of lymphoblastoid growth in large tight clumps. In contrast, long-term BL cell lines of malignant origin are more heterogeneous and display any one of a range of cell surface and growth phenotypes (Favrot *et al.*, 1984; Rowe,M. *et al.*, 1985; Ehlin-Henriksson *et al.*, 1985). Recently our prospective studies have shown that this apparent tumour cell heterogeneity actually arises *in vitro* (Rowe,M. *et al.*, 1985; Rooney *et al.*, 1986); thus all BL biopsies examined, together with their derived cell lines in very ear-

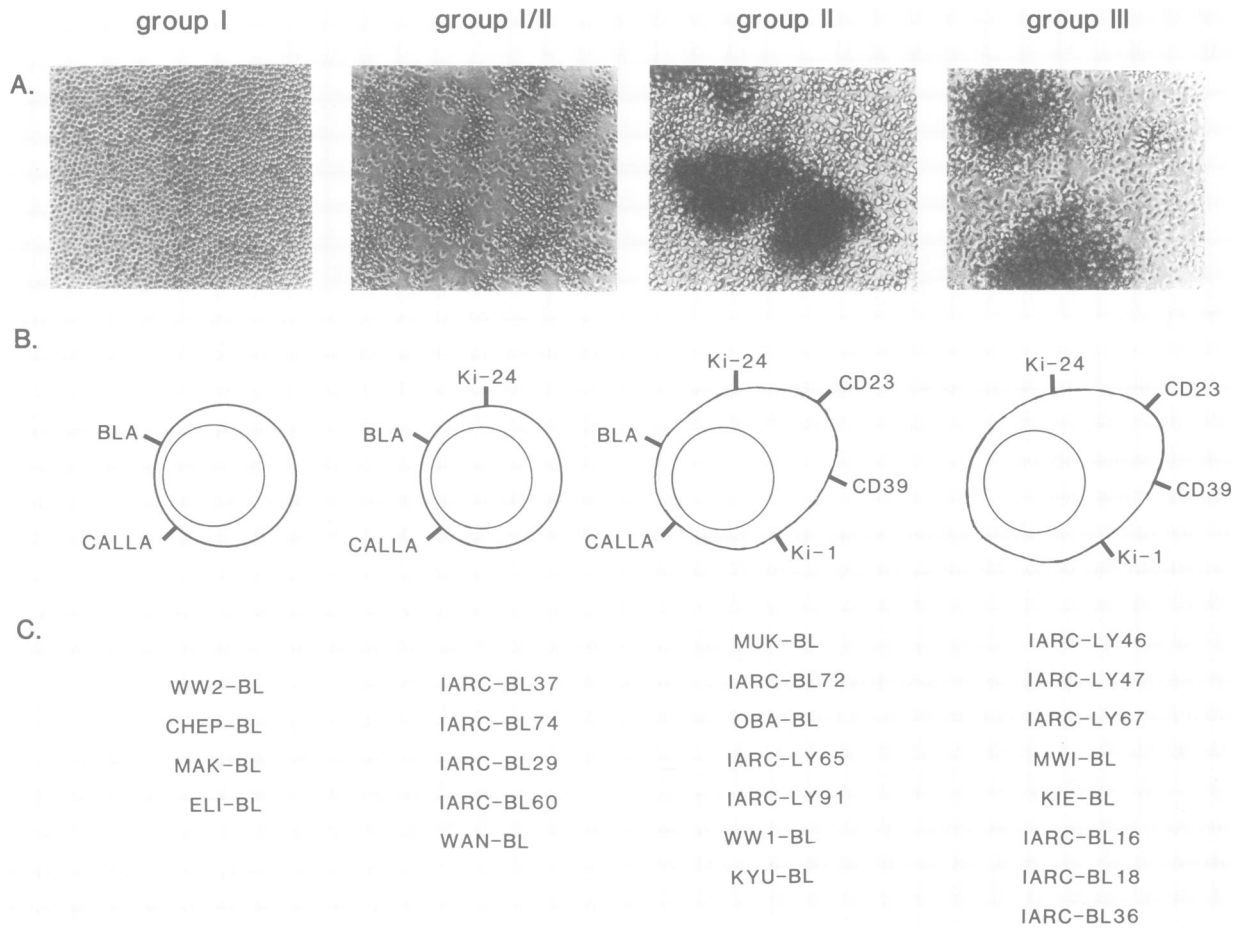

Fig. 1. Phenotypic grouping of 24 EBV-positive BL cell lines studied within 20–50 *in vitro* passages of their establishment from tumour biopsies. The groups can be distinguished in terms (**A**) of cell growth pattern and (**B**) of cell surface markers; each BL cell line was assigned as shown (**C**). Note that group II is the most heterogeneous with respect to cell surface marker expression; some group II lines (e.g., MUK-BL) expressed only CD23 in addition to the group I/II markers whereas other lines (e.g., KYU-BL) expressed CD23, CD39 and Ki-1.

ly passage, displayed a homogeneous cell surface phenotype which was quite distinct from that of LCLs. On subsequent passage the individual BL cell lines, whilst retaining their cytogenetic markers of malignant origin, showed different degrees of progression towards an LCL-like phenotype and growth pattern, such that phenotypic differences between the lines were quickly generated.

In the present report we show that this phenotypic heterogeneity amongst BL cell lines is associated with differences in their pattern of EBV latent gene expression. Specifically, BL biopsy cells and those derived cell lines which retain the biopsy cell phenotype *in vitro* do not express detectable amounts either of EBNA 2 or LMP, whilst the progression of a BL cell line towards a more LCL-like phenotype is linked with the appearance of these 'transforming' proteins.

Results

Phenotypic heterogeneity of BL cell lines

Twenty four BL cell lines, recently established from EBV-positive BL biopsies and displaying the specific chromosomal translocation indicative of their malignant origin, (Lenoir *et al.*, 1985; Rooney *et al.*, 1986) were studied between their 20th and 50th *in vitro* passage. Since almost all the lines had by this stage reached the limit of their *in vitro* progression and had become phenotypically stable, they could be assigned to one of the four groups shown in Figure 1 on the basis (i) of their growth pattern and (ii) of their reactivity with a selected panel of monoclonal antibodies against B lineage-associated cell surface markers (Rowe,M. *et al.*, 1985).

BL cell lines in group I grew as a single cell carpet and had retained the original BL biopsy cell surface phenotype, with expression of the common acute lymphoblastic leukaemia antigen (cALLA, CD10) and of the BL-associated glycolipid antigen (BLA) in the absence of those B cell 'activation' markers consistently present on all LCLs, i.e., the Ki-24 antigen, the 45 kd CD23 protein, the 80 kd CD39 protein, and the 110 kd Ki-1 antigen. Certain lines, designated group I/II, showed limited progression from the group I phenotype to grow in small loose clumps with accompanying expression of the Ki-24 'activation' antigen in addition to cALLA and BLA. In contrast, other BL lines showed more marked progression leading to growth in large clumps, a more lymphoblastoid cell morphology and the expression of further cellular 'activation' antigens CD23, CD39 and Ki-1. As shown in the figure, such lines either stabilised in group II or progressed even further, losing their original cALLA and BLA markers to assume a group III cell surface phenotype essentially similar to that of all LCLs. Cloning of cells from early passage cultures confirmed that these group II/III lines did indeed arise by phenotypic progression *in vitro* of cells originally

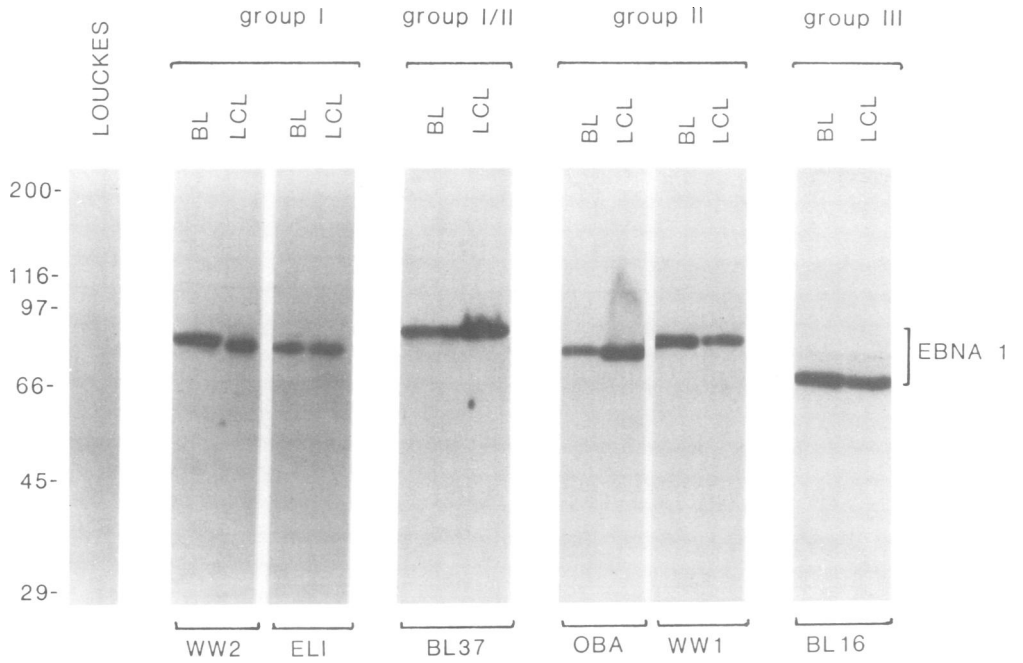

Fig. 2. Levels of EBNA 1 expression in BL cell lines, and in paired LCLs carrying the same EBV isolates. EBNA 1 was detected by SDS−PAGE and immunoblotting of cell extracts with a human serum (MO) having high anti-EBNA 1 reactivity, no reactivity against other EBNA species and unusually low reactivity against antigens of the virus productive cycle. The various BL/LCL pairs are arranged according to the phenotype group of the BL cell line; Louckes is an EBV-negative BL cell line included as a control.

expressing group I cell surface markers and were not generated by selective overgrowth from a rare phenotypic variant present within the original tumour population *in vivo* (Rooney *et al.*, 1986). All of the BL lines, irrespective of their phenotype group, retained the karyotypic markers and the monotypic pattern of immunoglobulin expression characteristic of the original biopsy.

EBV latent gene expression in relation to BL cell phenotype

In these experiments, each of the available BL cell lines was examined for EBV latent gene expression alongside a matching LCL, which had been generated by *in vitro* transformation of normal B cells using virus rescued from the BL line itself (see Materials and methods). The LCL thus served as a positive control indicating how each particular BL-derived virus isolate was expressed in the normal lymphoblastoid cell environment.

The various BL cell lines, and their matching LCLs, were first examined for EBNA 1 expression by immunoblotting using two particular human reference sera (MO, DR) chosen for their strong anti-EBNA 1 reactivity, their lack of reaction with other EBNA species, and their low antibody titres against antigens of the virus productive cycle. Figure 2 shows immunoblots, probed with the MO serum, of extracts from 6 BL cell lines representative of the spectrum of BL phenotypes *in vitro*. Every BL line expressed the EBNA 1 protein in easily detectable amounts, irrespective of phenotypic grouping. Note that whilst the molecular size of the antigen varied from one BL line to another, the matching BL/LCL pairs carrying the same EBV isolate always expressed EBNA 1 proteins of the same size. To further confirm the specificity of the immunoblotting reaction, control extracts from EBV-negative cell lines (in Figure 2, the 'sporadic' BL cell line Louckes) showed no EBNA 1 band.

The complete panel of BL/LCL pairs was then analysed for EBNA 2 expression using selected human sera with reactivity predominantly directed either against EBNA 2A (PK, RM, CH) or against EBNA 2B (AM). The pattern of results obtained is again illustrated using the same panel of 6 representative BL/LCL pairings, the immunoblots being shown in Figure 3. The first point to note is that each of the LCL extracts reacted preferentially with one of the two types of sera, i.e. either they contained a 85 kd EBNA 2A protein (BL37, OBA and WW1 LCLs) or a 75 kd EBNA 2B protein (WW2, ELI, and BL16 LCLs), reflecting the EBNA 2 type of their particular EBV isolate. More importantly, it is clear from Figure 3 that not all BL cell lines expressed an EBNA 2 protein. Thus, both BL cell lines in phenotype group I (WW2, ELI) and the cell line in group I/II (BL37) showed no evidence of an EBNA 2 band, whereas the relevant EBNA 2 gene product was found in the BL cell lines with a more LCL-like group II (OBA, WW1) or group III (BL16) phenotype. Even prolonged exposure of the gels failed to reveal any EBNA 2 expression in the above group I and group I/II BL lines. Note that both of the sera used here (PK serum absorbed with P_3HR_1 cell extract for EBNA 2A, and AM serum for EBNA 2B) lack anti-EBNA 1 reactivity so that the EBNA 1 proteins known to be present in all 6 BL cell line extracts (see Figure 2) are not detected. The additional bands detected by AM serum in certain cell lines (e.g., WW2 LCL, ELI LCL, BL16) are proteins of the virus lytic cycle and reflect the high virus producer status of these particular lines.

The corresponding analysis of LMP expression across the complete spectrum of BL/LCL pairs again revealed differences between BL cell lines which appeared to be related to their degree of phenotypic progression. Here the serological probe was a pool of 4 monoclonal antibodies (CS.1−4, Rowe,M. *et al.*, 1987) raised against a bacterial fusion protein containing the carboxy half of the LMP molecule (Hennessy *et al.*, 1984). This pooled reagent detected a major LMP band in cell extracts of every LCL examined, varying in size from 57 to 66 kd depending on the virus isolate and sometimes accompanied by minor lower

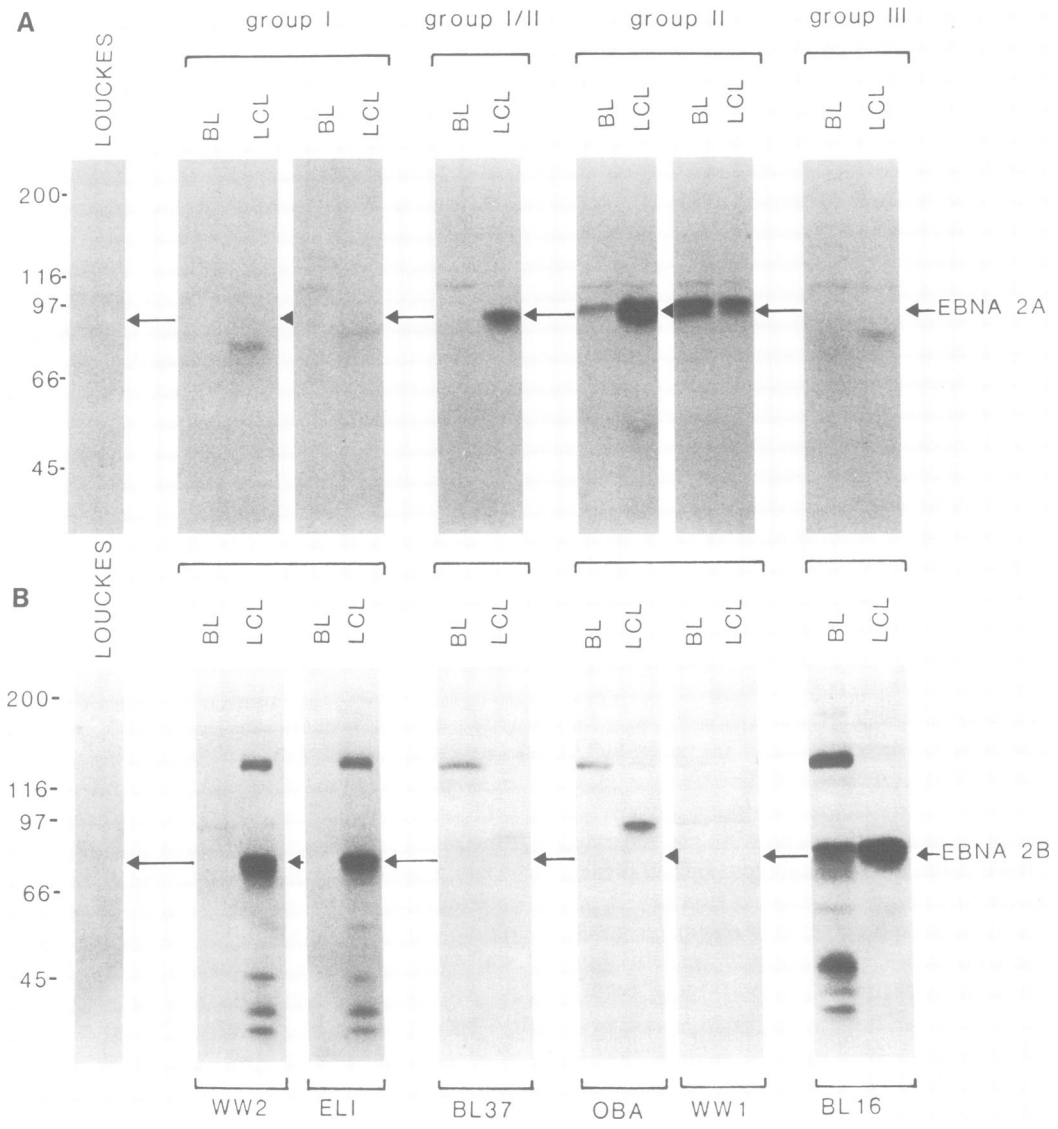

Fig. 3. Levels of EBNA 2 expression in BL/LCL pairs (arranged as in Figure 2) as detected by SDS−PAGE and immunoblotting of cell extracts. (**A**) EBNA 2A expression detected with a human serum (PK, absorbed with P_3HR_1 cell extract) having high anti-EBNA 2A and no anti-EBNA 1 reactivity; this preferentially recognises the 85 kd EBNA 2A protein encoded by the BL37, OBA and WW1 (type A) virus isolates. (**B**) EBNA 2B expression was detected with a human serum (AM) having high anti-EBNA 2B and no anti-EBNA 1 reactivity; this preferentially recognises the 75 kd EBNA 2B protein encoded by the WW2, ELI and BL16 (type B) virus isolates. Note that both sera are not completely type-specific in anti-EBNA 2 reactivity; there is weak cross-reactive recognition of EBNA 2A by AM serum and of EBNA 2B by PK serum. The additional bands seen in some lanes with AM serum (e.g., WW2 LCL, ELI LCL, BL16) represent antigens of the virus productive cycle and reflect the high virus producer status of these particular lines.

molecular weight (50−55 kd) species. The antibodies also crossreacted in immunoblots with a 43−44 kd doublet of uncharacterised normal cell proteins found in every cell line examined (Rowe,M. *et al.*, 1987); in the present work this doublet served as a useful confirmation that similar amounts of protein had indeed been loaded onto each gel track. Figure 4 presents the results obtained when cell extracts from the same 6 BL/LCL pairings as used in Figures 2 and 3 were probed with these anti-LMP monoclonal antibodies. Once again the BL cell lines which had progressed to a group II or group III phenotype expressed an LMP molecule of the same size as seen in the corresponding LCL, whereas the group I and group I/II BL cell lines showed no detectable expression of the protein.

Table I provides a summary of the EBNA 1, EBNA 2 and LMP immunoblotting results for each of the 24 BL cell lines analysed. The lines are arranged according to phenotype group to stress the clear relationship which exists between cell surface/growth phenotype of the BL cell and the pattern of latent viral gene expression. Whilst the EBNA 1 protein was found in every EBV-carrying BL cell line examined, all four group I BL cell lines and a proportion of those in group I/II gave no detectable expression either of EBNA 2 or of LMP; in contrast, these two proteins were demonstrably expressed in all 7 group II and in all 8 group III BL cell lines. This summary table was compiled after the analysis of at least two separate protein extracts from each cell line, each extract being probed both for EBNA 2 and for LMP on at least two independent occasions, where necessary with prolonged autoradiographic exposures of gels. The clear differences in patterns of EBV latent gene expression between individual lines were reproducibly observed throughout.

We specifically addressed the question of whether such differences were reflected at the level of RNA transcription using

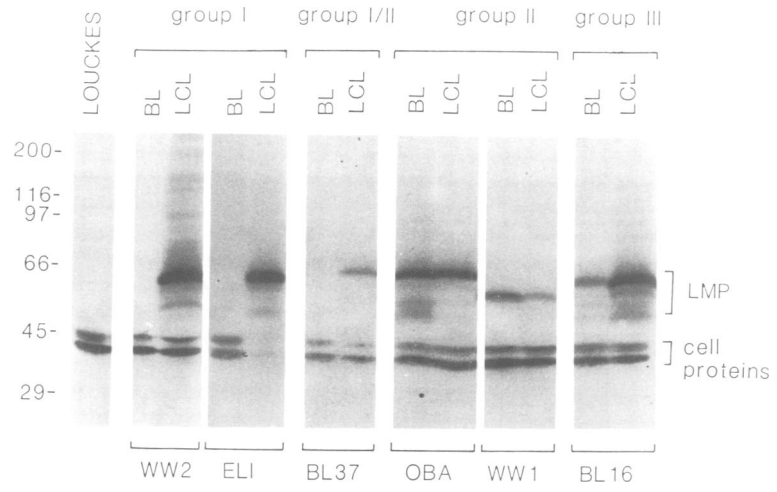

Fig. 4. Levels of LMP expression in BL/LCL pairs (arranged as in Figure 2) as detected by SDS−PAGE and immunoblotting of cell extracts with a pool of four anti-LMP MAbs (CS1−4). LMP is recognised as a major band, varying in size from 57−66 kd depending on the virus isolate, and sometimes accompanied by minor 50−55 kd species (Rowe,M. *et al.*, 1987). The MAbs also cross-react with a 43−44 kd doublet of cellular proteins present in every cell extract including that from the EBV-negative control cell line Loucks.

Table I. Detectable EBV latent gene expression in BL cells grouped according to cell growth/surface phenotype

Group I				Group I/II				Group II				Group III			
Cell line	EBNA 1	EBNA 2	LMP	Cell line	EBNA 1	EBNA 2	LMP	Cell line	EBNA 1	EBNA 2	LMP	Cell line	EBNA 1	EBNA 2	LMP
WW2-Bl	+	−	−	BL37	+	−	−	MUK-BL	+	+	+	LY46	+	+	+
ELI-BL	+	−	−	BL74	+	−	−	BL72	+	+	+	LY47	+	+	+
MAK-BL	+	−	−	BL29	+	−	+	OBA-BL	+	+	+	LY67	+	+	+
CHEP-BL	+	−	−	BL60	+	+	−	LY65	+	+	+	MWI-BL	+	+	+
				WAN-BL	+	+	+	LY91	+	+	+	KIE-BL	+	+	+
								WW1-BL	+	+	+	BL16	+	+	+
								KYU-BL	+	+	+	BL18	+	+	+
												BL36	+	+	+

Summary of results from phenotypically-grouped BL cell lines studied between passage 20 and 50 *in vitro*. At least two independent protein extracts were made from each BL cell line, and from each LCL carrying the corresponding EBV isolate, and were assayed as described.

BL cell lines where, by immunoblotting, LMP expression in the BL lines was either undetectable (BL74, group I/II) or trace (BL29, group I/II) or comparable to the LCL (BL72 and WW1-BL, group II). The BL18 line was also included here as these cells showed a comparatively low level of expression for a group III BL line (Rowe,D. *et al.*, 1986). As shown in Figure 5, when polyA+RNA from these lines was examined for LMP message by Northern blotting, the relevant RNA band was detectable in BL72 and WW1-BL cells and at a low level in BL18 cells, but could not be seen in BL74 or BL29 cells; control LCLs did express the message (Figure 5B). Differences in LMP expression at the protein level therefore do appear to be reflected at the transcriptional level. The result with BL29 suggests that detection of LMP by immunoblotting is more sensitive than by Northern analysis. At the same time it was interesting to note that all BL and LCL lines tested contained the small non-polyadenylated EBER RNAs (Rymo, 1979), suggesting that expression of these non-translated species is independent of host B cell phenotype (Figure 5A).

Prospective studies on individual BL cell lines

In BL cell lines which progress to a group II or group III phenotype, the change occurs so rapidly after the initiation of *in vitro* growth (Rowe,M. *et al.*, 1985) that it is difficult to monitor for changing patterns of virus gene expression. Within the present BL cell panel, however, there were two lines (WW2, CHEP) which spontaneously moved from group I into group I/II with an accompanying change to growth in loose clumps between passage 65 and 85.

Figure 6 presents data comparing the expression of latent virus proteins in early passage cultures of these lines (passage 10−20) with that seen after their eventual phenotypic progression. In the WW2 BL cell line, which was clearly EBNA 2- and LMP-negative in early passage, expression of LMP (but not EBNA 2) was detectable following progression into group I/II. A similar degree of phenotypic change occurring in later passages of the CHEP-BL cell line was accompanied by detectable expression both of EBNA 2 and, at very low levels, of LMP.

EBV latent gene expression in BL biopsy cells

The inference from the above work on cultured BL cell lines was that EBV latent gene expression was unusually restricted in cells whose surface phenotype (group I) mirrored that shown by BL tumour cells *in vivo* (Rooney *et al.*, 1986). A final set of experiments therefore addressed the crucial question of EBV latent gene expression in BL biopsy cell themselves. Although opportunities for such a study were limited, cryopreserved preparations of three biopsy cell suspensions, from patients CHEP, WAN and MUK were available and were already known from phenotypic analysis to be composed largely of viable tumour

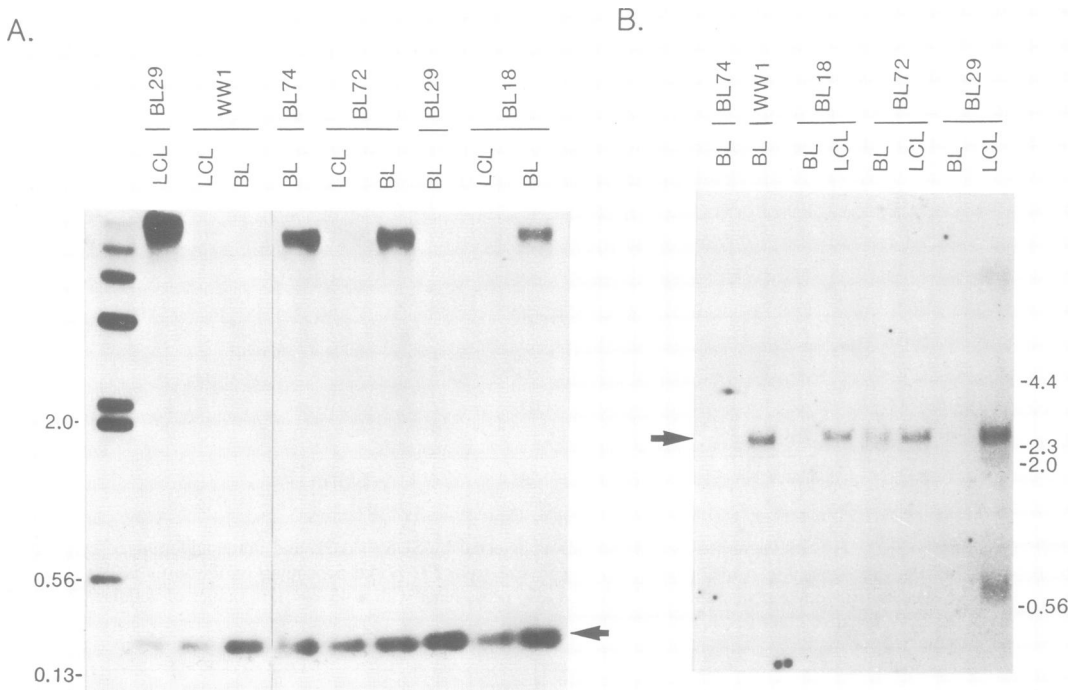

Fig. 5. Levels (A) of EBER RNAs and (B) of LMP RNA in selected BL and LCL lines as detected by Northern blotting. (**A**) Tracks containing polyA- RNA from the cell lines were probed with the Bam H1C fragment of B95.8 EBV DNA (nucleotides 3994−13215). The EBER RNAs, which co-migrate on this type of gel, are arrowed. Markers (right hand track) were an end-labelled Hind III digest of phage λ DNA. (**B**) Tracks containing polyA+ RNA from the cell lines were probed with pDH3 (B95.8 nucleotides 166481−169033). The LMP RNA is arrowed. Markers (not shown) were as above. The filter was subsequently rehybridised with an actin RNA probe to ensure that all tracks contained equivalent amounts of RNA and that there was no degradation.

cells with less than 10% infiltration by normal lymphocytes (Rooney *et al.*, 1986).

The relevant immunoblots of extracts from biopsy cells, from the derived BL cell line and from its paired LCL are shown for all three tumours in Figure 7. EBNA 1 could be detected in all 3 biopsies, although in lower amounts than was seen in the corresponding BL cell line (Figure 7A) even though the same amount of total protein was present in each sample. In contrast, none of the biopsy cell extracts gave detectable expression either of EBNA 2 (Figure 7B) or of LMP (Figure 7C) even in those cases where the proteins could be readily detected in the derived BL cell lines after phenotypic progression *in vitro*. These results were confirmed in a second series of immunoblots using these same biopsy cell extracts, and a similar pattern of latent gene expression was observed in the one experiment which could be performed on biopsy material from a fourth BL patient, OBA.

Discussion

EBV-transformed LCLs generated from normal B cells *in vitro* constitutively express a limited set of viral proteins, the so-called 'latent' virus gene products (reviewed Dambaugh *et al.*, 1986), and it has been widely assumed that this also reflects the pattern of virus gene expression present in EBV genome-positive BL cell lines. A preliminary examination of a small number of BL/LCL pairs demonstrated that novel patterns of EBV gene expression could exist in BL cells (Rowe,D. *et al.*, 1986). The present study has examined this phenomenon using a much wider panel of BL cell lines whose *in vitro* life history and whose degree of phenotypic progression (Figure 1) have been carefully monitored (Rowe,M. *et al.*, 1985; Rooney *et al.*, 1986). The results (Figures 2−5, Table I) show that cell lines which stably retained the original BL cell phenotype (group I) and which grew as a single cell carpet *in vitro*, expressed EBNA 1 but did not express detectable amounts of either EBNA 2 or LMP. In contrast those lines which had progressed to phenotype groups II and III and which had assumed a more LCL-like growth pattern showed detectable expression of all three latent viral gene products. Furthermore our most recent results using a monoclonal antibody probe to the EBNA 3 protein (Rowe,M. unpublished observations) and those of Dillner *et al.* (1986) using a rabbit antiserum to LP (designated EBNA 5 by these authors) indicate that these other latent viral gene products may also show similar phenotype-dependent expressions.

The inference that *in vitro* progression is accompanied by a broadening of EBV latent gene expression was confirmed in the two examples where progression could be followed prospectively. Both involved lines moving in late passage from group I to group I/II, a change associated with the appearance in one case (WW2) of LMP, and in the other case (CHEP) of both EBNA 2 and LMP (Figure 6).

Although most BL lines studied (Table I) were either negative both for EBNA 2 and LMP or positive for the two antigens, the results from BL29 and BL60 suggested that expression of these two proteins was not strictly co-regulated. Again, in preliminary work with a separate BL74 culture (Rowe,D. *et al.*, 1986), there was evidence of EBNA 2 expression in the absence of LMP.

Given the finite sensitivity of the immunoblotting assay, it is possible that the phenotype-related differences in EBV latent gene expression summarised in Table I are quantitative rather than absolute. Nevertheless for the four group I BL cell lines studied, neither an EBNA 2 nor an LMP band could be detected in immunoblots under conditions where the corresponding LCL extracts could be diluted 500 to 1000-fold and still gave detectable

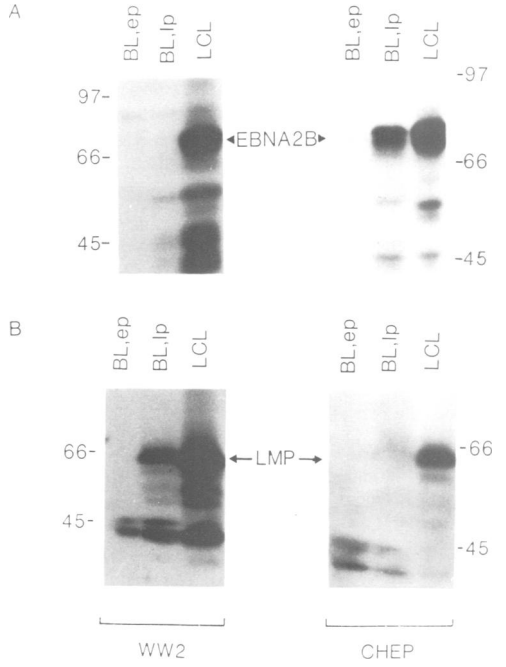

Fig. 6. Levels (A) of EBNA 2 expression and (B) of LMP expression in early passage (e.p., passage 20–25) and in late passage (l.p., passage 65–85) cultures of the WW2- and CHEP-BL cell lines, compared in each case with expression of the same proteins in LCL cells carrying the same virus isolates (both of type B). The figure shows immunoblots probed (**A**) with the AM serum for EBNA 2B, and (**B**) with MAbs CS1-4 for LMP.

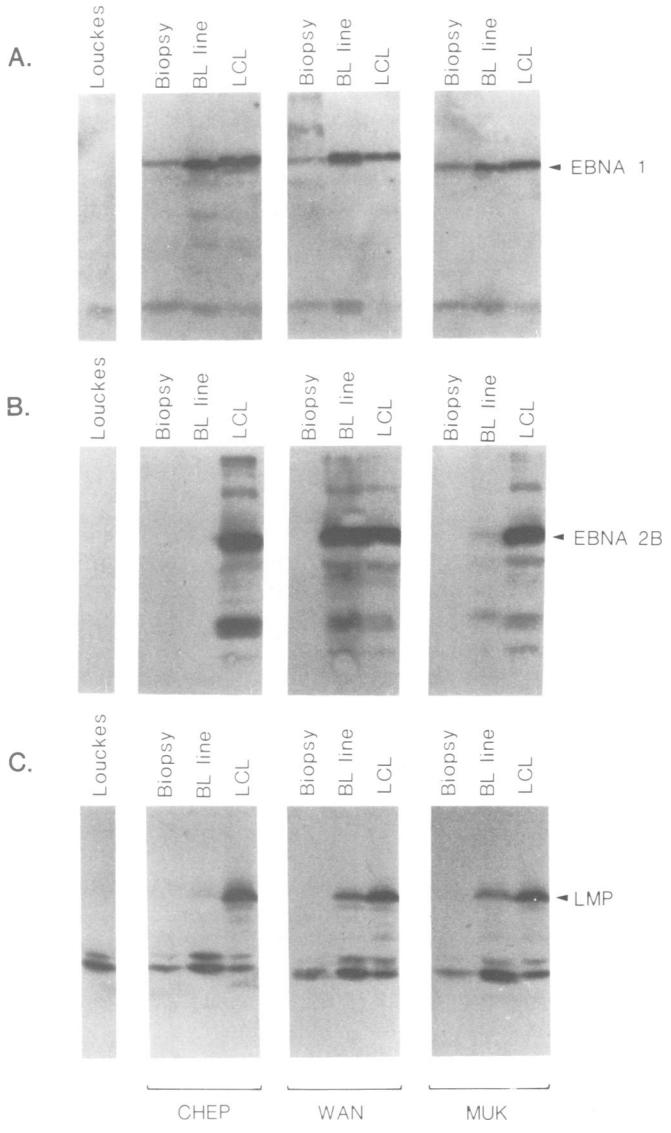

Fig. 7. Levels (**A**) of EBNA 1 expression, (**B**) of EBNA 2 expression and (**C**) of LMP expression in CHEP, WAN and MUK BL biopsy cells, in the derived BL cell lines and in the paired LCLs carrying the same virus isolates (all of type B). The figure shows immunoblots probed (A) with the DR serum for EBNA 1, (B) with the AM serum for EBNA 2B, and (C) with MAbs CS1-4 for LMP. Coomassie blue staining of the BL biopsy lanes confirmed that equivalent levels of protein had been loaded as in the adjacent lanes loaded with cell line extracts (data not shown); note also that cross-reactivity of the DR serum against a low mol. wt cellular protein (see A) and of the MAbs CS1-4 against a doublet of cellular protein (see C) was observed with the BL biopsy extracts just as with the cell line extracts. The BL cell line extracts used in each case were from passage 20–50 cultures phenotyped as in Figure 1, with the exception of the CHEP-BL extract in C which was from the same late passage culture as described in Figure 5.

signals for both viral proteins. Recently more sensitive immunoprecipitation methods with the CS.1–4 monoclonal antibodies confirmed the LMP results (M.Rowe and L.S.Young, unpublished observations). Clearly if there is EBNA 2 and/or LMP expression in group I BL cell lines, then it is very dramatically reduced relative to that shown by the same virus isolate in a lymphoblastoid cell environment. The mechanism by which EBNA 2 and LMP are down-regulated in BL cell lines appears to operate at the RNA level rather than by translational control. This could be due to control of transcription, splicing and/or RNA stability.

BL biopsy cells gave results which were similar to those seen with group I BL cell lines, that is expression of EBNA 1 but no evidence of either EBNA 2 or LMP (Figure 7). However, the level of EBNA 1 was itself comparatively low in the biopsy cell extracts, even though the extracts were prepared from cell suspensions composed almost exclusively of tumour cells, showed no evidence of protein degradation by Coomassie Blue staining, and were loaded onto the gel to give equivalent levels of protein per track as for the adjacent cell line extracts.

Are the different patterns of EBV gene expression in cultured BL cell lines a cause or a consequence of the cells' phenotypic progression? Most evidence to date argues that EBV causes the progression. EBV genome-negative BL cell lines show remarkable phenotypic stability, many remaining in group I or group I/II even after several years in culture (Rowe,M. *et al.*, 1986). Conversion of such virus-negative lines to EBV genome-positivity by *in vitro* infection induces the cells to grow in large clumps and to express the additional activation antigens of phenotype group II (Rowe,M. *et al.*, 1986). Furthermore in recent experiments where individual EBV genes have been introduced by recombinant retrovirus transfer into EBV-negative BL cell lines (Wang *et al.*, 1987), expression of the EBNA 2 gene caused the cells to grow in clumps and up-regulated the cellular activation antigen, CD23, thus mimicking some of the crucial events of 'lymphoblastoid' progression. Our working hypothesis, therefore, is that differences in the degree of phenotypic progression shown by individual EBV-positive BL cell lines reflect underlying differences in their patterns of virus gene expression.

The observation of a highly restricted pattern of EBV latent gene expression in BL tumour cells defines what is essentially

a new type of EBV:cell interaction. Such results have important implications for current thinking about the possible role of the virus in the pathogenesis of this tumour. Two existing pieces of evidence argue strongly against the association of EBV with BL being that of a casual passenger. Firstly, the virus is not found in any other B cell lymphomas (with the exception of those occurring in immunosuppressed patients) even though such tumours occur in virus-infected individuals and frequently express the C3d/EBV receptor molecule (Stein, 1978). Secondly, spontaneous outgrowth assays on blood from malaria carriers resident in BL endemic areas indicate that only a very small proportion of their total B cell pool (certainly $<1\%$) is detectably infected with EBV (Moss *et al.*, 1983); chance infection of the malignant B cell clone *in vivo* is therefore very unlikely to account for the presence of the virus in $>95\%$ endemic Burkitt tumours. We would argue that the virus is indeed causally implicated in the pathogenesis of EBV-positive BL, but that the malignant clone which finally emerges *in vivo* may by that time be independent of at least two latent viral gene products, EBNA 2 and LMP, for its continued growth. Activation of the c-myc proto-oncogene by chromosomal translocation is clearly important by this stage (Klein, 1983). The consistency with which the EBV genome is still retained in BL tumour cells during *in vivo* outgrowth suggests that the virus nevertheless continues to provide some growth advantage. Since, of the known latent viral proteins, only EBNA 1 has as yet been detected consistently in BL cells, it will be important to search for functions of this protein other than its already described role in virus genome maintenance (Yates *et al.*, 1984, 1985; Rawlins *et al.*, 1985).

The latent viral gene products EBNA 2 and LMP appear to play essential roles in virus-induced growth transformation of normal B cells *in vitro*, but their role in the oncogenic sequence leading to BL remains to be established. In this context, if the primary role of EBV is to induce polyclonal B cell proliferation and thus to expand the pool of cells potentially at risk of subsequent chromosomal translocation (Klein 1979, 1983), then this implies a requirement for the full spectrum of latent virus proteins at an early stage of the oncogenic sequence. Alternatively, if EBV contributes to lymphomagenesis by infecting a rare cell which has already undergone the translocation step (Lenoir and Bornkamm, 1986), thereby enhancing that cell's existing growth advantage, the 'transforming' functions of EBNA 2 and LMP may not be obligatory for outgrowth. Either way, virus-specific T cell surveillance, which normally serves to control the numbers of EBV-infected B cells *in vivo* (Rickinson *et al.*, 1981), would exert a selection pressure favouring the outgrowth of a malignant clone in which any non-essential virus latent gene products are not expressed. We have indeed shown that the broadening of EBV latent gene expression which one sees with phenotypic progression in cultured BL cell lines is associated with increased sensitivity to virus-specific T cell detection (Rowe,D. *et al.*, 1986). The same process occurring *in vivo* would thus render the tumour cells sensitive to this type of immune control.

Materials and methods

Cell culture

All cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% foetal calf serum from selected batches. Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in humidified air.

BL biopsies and derived cell lines

The BL cell lines used in these experiments were established from fresh EBV genome-positive tumour biopsies either in one of our laboratories (Department of Cancer Studies, Birmingham) or by Dr G.M.Lenoir (International Agency for Research on Cancer, Lyon, France) or Dr D.J.Moss (Queensland Institute of Medical Research, Brisbane, Australia), both of whom then kindly provided the lines in early passage. Full details of the establishment and characteristics of these lines are given elsewhere (Rooney *et al.*, 1984, 1986; Lenoir *et al.*, 1985); all were EB virus genome-positive and contained characteristic chromosome translocations indicative of their malignant origin. Biopsy cells used directly for experimental work were from viable cell suspensions in which $>90\%$ cells showed the characteristic surface phenotype of malignant BL cells.

Establishment of LCLs using BL-derived strains of EBV

Each of the above BL cell lines was treated for 3 days with 20 ng/ml TPA in order to induce infectious virus production. The cells were then washed thoroughly and inactivated by exposure to 4000 rads X-rays prior to co-cultivation with peripheral blood mononuclear cells from an EBV antibody-negative healthy donor. These co-cultures were set up in the presence of cyclosporin A at a concentration of 0.1 μg/ml in order to avert any coincidental T cell activation *in vitro* (Rickinson *et al.*, 1984), and were thereafter re-fed weekly with a half-change of medium (without cyclosporin A) until foci of virus-transformed B cells could be transferred out of the co-culture wells and used to establish an LCL.

Antisera and monoclonal antibodies

The human sera used in immunoblots to detect EBNA 1 (MO, DR, PK), EBNA 2A (PK, CH), and EBNA 2B (AM) were from patients with chronic active EBV infections (CH, AM, PK) and from healthy EBV seropositive individuals (DR, MO). These sera were selected for their strong reactivity in immunoblots against the antigen in question, and their relatively weak reactivities against other EBV antigens, particularly those of the virus productive cycle. In some experiments the PK serum, which recognises both EBNA 1 and EBNA 2A in immunoblots, was selectively depleted of anti-EBNA 1 reactivity by absorption with P_3HR_1 cells, a BL cell line with a deletion in the region of DNA coding for EBNA 2 (Bornkamm *et al.*, 1982). P_3HR_1 cells were washed twice in Dulbecco's phosphate-buffered saline (PBS), resuspended in PBS at 10^9 cells in 5 ml, sonicated, boiled for 2 min, and sonicated again; this cell extract was incubated with 0.5 ml of PK serum for 5 h and then the adsorbed serum was clarified by centrifugation at 20 000 *g* for 45 min at 4°C.

LMP was detected using a pool of 4 murine monoclonal antibodies (CS.1−4) described in detail elsewhere (Rowe,M. *et al.*, 1987) and raised to a bacterial fusion protein containing 189 amino acids of the carboxy half of the LMP (Hennessey *et al.*, 1984). This pooled reagent recognises at least 3 separate determinants on the LMP molecule and has detected the protein encoded by every EBV isolate thus far tested.

SDS−PAGE and immunoblotting

Cells were washed once in PBS, and solubilised by sonication in sample buffer. Cells were solubilised at a concentration of 10^6 cells/20 μl sample buffer (0.05 M Tris-buffer, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue), sonicated briefly to reduce the viscosity, and then boiled for 3 min. Samples corresponding to $1-2 \times 10^6$ cells per track were separated by discontinuous gel electrophoresis using a stacking gel of 5% acrylamide and a resolving gel of either 10% or 7.5% acrylamide (Laemmli, 1970), transferred onto nitrocellulose at a current of 150 mA for 5 h at 4°C, and probed with antibody and radioiodinated staphylococcal protein A by the following method adapted from Burnette (1981). The blotted filters were incubated for 2 h with PBS containing 5% skimmed milk (PBS−milk) to block non-specific binding sites before incubating with antibody for 16 h at 4°C; EBNA 1 and EBNA 2 were detected using immune human sera diluted 1:100 in PBS−milk, and LMP was detected using a pool of monoclonal antibodies diluted 1:15 in PBS−milk. After incubation with the primary antibody, the filters were washed with PBS−0.1% Tween 20. In experiments where LMP was being assayed using monoclonal antibodies, the filters were then incubated for 1 h at room temperature with a rabbit anti-mouse Ig (Dakopatts), diluted 1:5000 in PBS−milk, and washed again in PBS−Tween. No second-step antibody was used for detection of EBNAs using human sera. Specifically bound antibody was detected by incubating for 2 h with ^{125}I-labelled staphylococcal protein-A (Amersham) diluted to 0.1 μCi/ml to PBS−milk. Following a final wash in PBS−Tween, the filters were dried and subjected to autoradiography for 1−5 days with an intensifying screen. Mol. wt determinations were made using protein standards (Sigma) which had been prestained with Remazole brilliant blue according to the method of Griffith (1972).

Northern blotting

Cytoplasmic RNA was prepared (Farrell *et al.*, 1979) from various cell lines and fractionated on oligo dT cellulose (Aviv and Leder, 1972). 1 μg samples of RNA were glyoxylated (McMaster and Carmichael, 1977), electrophoresed on 1% agarose gels and blotted to nitrocellulose filters (Thomas, 1980). Filters were hybridised with nick translated probes (Rigby *et al.*, 1977).

Cell surface phenotyping of BL cells

Characterisation of the cell surface phenotype of BL cell lines was performed by indirect immunofluorescence upon viable cells using selected monoclonal an-

tibodies to 6 separate B lineage-associated antigens exactly as described elsewhere (Rooney *et al.*, 1986). Of these antibodies two reacted consistently with BL biopsy cells: J5, specific for cALLA (Ritz *et al.*, 1980), and 38.13 specific for the glycolipid BLA (Wiels *et al.*, 1981). The other four antibodies defined antigens constitutively expressed on all LCLs: Ki-24, specific for Sternberg-Reed cell-associated Ki-24 antigen (Stein *et al.*, 1983); MHM6, specific for the 45 kd B cell activation antigen designated CD23 (Rowe,M. *et al.*, 1982); AC2, specific for the 80 kd lymphocyte-activation antigen designated CD39 (Rowe,M. *et al.*, 1982); and Ki-1, specific for a 110 kd Sternberg-Reed cell-associated Ki-1 antigen (Schwab *et al.*, 1982).

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